

Part I. Identification and Characterization of a cDNA for 1-Aminocyclopropane-1-Carboxylate Synthase from Apices of Cucumber Plants.

Introduction

It has been suggested that sex expression in flowers of cucumber plants is regulated by levels of ethylene at the apex. Rudich *et al.* (1972, 1976) reported that a high correlation existed between the evolution of ethylene from apices and the formation of female flowers. More ethylene was evolved from apices of the gynoeocious than from those of the monoecious type (Rudich *et al.* 1972). Cucumber plants grown under short day conditions which promote femaleness evolved more ethylene than those grown under long day conditions (Rudich *et al.* 1972). Inhibitors of ethylene action and ethylene biosynthesis suppress female flower development (Beyer 1976, Atsmon and Tabbak 1979). Furthermore, it was reported that the activity of ACC synthase at the apex was higher in gynoeocious plants than in monoecious plants (Trebitsh *et al.* 1987).

Ethylene is a plant hormone which regulates many processes of plant growth, development and senescence (Abeles 1973). Ethylene is synthesized from methionine *via* S-adenosyl-L-methionine and ACC (Adams and Yang 1979). The enzymes catalyzing the individual steps of this pathway are S-adenosyl-L-methionine synthetase, ACC synthase and ACC oxidase, respectively. The biosynthesis of ethylene is primarily regulated at the level of the ACC synthase (Yang and Hoffman 1984). Many reports have shown that the ACC synthase enzyme is encoded by a multigene family and that they are differentially expressed in response to many factors (Huang *et al.* 1991, Nakagawa *et al.* 1991, Olson *et al.* 1991, Rottmann *et al.* 1991, Liang *et al.* 1992, Zarembinski and Theologis 1993, Destefano-Beltran *et al.* 1995)

The identification of the specific ACC synthase gene that is involved in the development of female flowers is important to understand

the role of endogenous ethylene in the regulation of sex expression. In this part, I describe the identification of a cDNA (*CS-ACS2*) for ACC synthase from the apices of cucumber plants and show that both the timing and the levels of expression of the *CS-ACS2* transcript were correlated with the development of female flowers at the node.

Materials and Methods

Plant Materials

The seeds of monoecious cucumber cultivars (*Cucumis sativus* L., cv. Ougonmegami 2-gou and cv. Shimoshirazu) and a gynoecious cucumber cultivar (*C. sativus* L., cv. Rensei) obtained from a local market. They were grown in soil-filled pots in a green house at 25 °C with 12 h of light per day. The apices were cut off seedlings just below the youngest leaf at indicated stages of growth, frozen immediately in liquid nitrogen, and stored at - 80°C prior to extraction of nucleic acids. For study of localization of expression of *CS-ACS2* transcript in the apex of a cucumber plant, the apices of 25-day-old Rensei and Shimoshirazu plants were prepared as described above, and floral buds and unexpanded leaves from the eighth node to the sixteenth node were excised from the apices under a light-microscope, frozen immediately in liquid nitrogen, and stored at - 80°C prior to extraction of nucleic acids.

Immature green fruits of cucumber (*C. sativus* L.) were purchased from a local market. Immature green fruits of cucumber were peeled and longitudinally cut in half, then transversely cut into 4 mm-thick slices. The slices were incubated with or without a solution of 0.5 mM IAA in 1 mM K-phosphate buffer, pH 7, at 25°C for 8 h as reported by Nakagawa *et al.* (1991). The treated slices were immediately frozen in liquid nitrogen and stored at -80°C prior to extraction of nucleic acids.

Isolation of RNA

Total RNA was extracted from the apices of cucumber seedlings

and the treated slices of cucumber fruits, respectively, as described by Prescott and Martin (1987), and then RNA was purified by precipitation in lithium chloride. Poly (A)+RNA was isolated from the total RNA with PolyATtract® mRNA isolation system III (Promega, Inc., Madison, WI, USA).

Isolation of cDNAs for ACC synthases

To isolate ACC synthase fragments, degenerate oligonucleotide primers homologous to conserved regions of ACC synthases were synthesized. The degenerate oligonucleotides ASP-1F, 5'-ATICA[A/G]ATGGGI[T/C]TIGCIGA[A/G]AA[T/C]CA-3' and ASP-2F, 5'-TT[T/C]CA[A/G]GA[T/C]TA[T/C]CA[T/C]GGI[C/T]TICC-3' and ASP-1R 5'-GTICCIA[A/G]IGG[A/G]TTIGAIGG[A/G]TT-3' correspond to the amino acid sequences of IQMGLAENQ and FQDYHGLP and NPSNPLGT, respectively. RT-PCR was performed with a GeneAmp RNA PCR Kit (Perkin-Elmer Japan Co. Ltd., Urayasu, Japan). ACC synthase gene fragment designated CS-ACS2 was amplified by RT-PCR using ASP-1F and ASP-1R as primers and total RNA isolated from apices of 20-day-old Ougonmegami 2-gou seedlings (the three-leaf stage). ACC synthase gene fragment designated CS-ACS3 was amplified by RT-PCR using ASP-1F and ASP-1R and total RNA isolated from auxin-treated immature cucumber fruits, then an aliquot of the primary amplification product was reamplified using ASP-2F and ASP-1R. ACC synthase gene fragment designated CS-ACS4 was amplified by RT-PCR using ASP-1F and ASP-1R and total RNA isolated from wounded immature cucumber fruits, then an aliquot of the primary amplification product was reamplified using ASP-2F and ASP-1R. The parameters for PCR were 50 cycles of heating at 94

°C for 1 min, at 55 °C for 2 min and at 72 °C for 3 min. The product of PCR was analyzed by gel electrophoresis on a 2% agarose and recovered with a Mermaid kit (BIO 101, Inc., La Jolla, CA., USA). The recovered product was cloned into the pCR™ II vector by the method described in the TA Cloning Instruction Manual (Invitrogen, San Diego, CA., USA).

Full-length cDNA encoding CS-ACS2 was amplified by RACE method. All reactions were performed with Marathon™ cDNA Amplification Kit by the method described in the Protocol and Reference Manual (CLONTECH Laboratories, Inc., CA., USA). The cDNA was synthesized from Poly (A)+RNA isolated from apices of 20-day-old Ougonmegami 2-gou seedlings and it was ligated to the Marathon cDNA adaptor. The 5' cDNA fragment was amplified by PCR using CS-ACS2 specific primer 1 (5'-GTAAGGAGTGGGGACAAGCAAAG-3') and the adaptor primer that was supplied in the kit as primers and the adaptor-ligated cDNA as a template. The 3' cDNA fragment was amplified by PCR using CS-ACS2 specific primer 2 (5'-GGAAGAAATTAGAGGTGGAAGAGC-3') and the Adaptor primer as primers and the adaptor-ligated cDNA as a template. The parameters for PCR were 30 cycles of heating at 94 °C for 30 sec, at 60 °C for 30 sec and at 68 °C for 4 min. The PCR products were cloned into the pCR™ II vector.

Sequencing of DNA

The cDNA inserts were excised with *EcoRI* from the pCR™ II vector and subcloned into pUC19. A series of deletion mutants were prepared by exonuclease III digestion followed by blunt-end ligation. DNA sequencing was performed by the dideoxy sequencing method (Sanger *et al.*

1977) using a Taq Dye Primer Cycle Sequencing kit (Perkin-Elmer Japan Co. Ltd.) and a DNA sequencer (model 377; Perkin-Elmer Japan Co. Ltd.).

Preparation of a cDNA Probe

Each of the cloned RT-PCR product was cleaved by *EcoRI* from the pCR[™] II vector that had been amplified in *Escherichia coli* (INV α F') and it was purified by gel electrophoresis and recovered. The cDNA was labeled with [α -³²P]dCTP by the random-priming method with a Multiprime[™] DNA labeling system (Amersham International plc, Amersham, Bucks., UK) and used as the probe.

Genomic DNA Gel Blot Analysis

Genomic DNA was extracted from 6-day-old etiolated cucumber hypocotyls followed by the method described previously (Okamoto *et al.* 1995). Two micrograms of DNA isolated from hypocotyls of the three cucumber cultivars (cv. Rensei, Ougonmegami 2-gou and Shimoshirazu) were separately digested with the restriction endonucleases *EcoRV* and *SacI*. The digests were fractionated on a 0.8% (w/v) agarose gel and transferred to a GeneScreen Plus membrane (Du Pont, Boston, MA, U.S.A.) as recommended by the manufacturer. The membrane was preincubated in 1 M NaCl, 1% SDS and 10% dextran sulfate (sodium salt) at 65 °C for 1 h. The denatured ³²P-labeled probe and denatured salmon-sperm DNA were then added to the prehybridization solution, and the membrane was incubated at 65 °C for 16 h. Post-hybridization washes were performed twice successively for 5 min each in 2x SSC (1x SSC is

0.15 M NaCl, 15 mM sodium citrate) at room temperature and once for 30 min with 2x SSC, 1% SDS at 65 °C. The washed membrane was subjected to autoradiography with an intensifying screen.

Expression Analysis by RT-PCR

Total RNA was isolated from the apices of 25-day-old seedlings of gynocious cucumber (cv. Rensei). RT-PCR was performed with a GeneAmp RNA PCR Kit (Perkin-Elmer Japan Co. Ltd., Urayasu, Japan) using ASP-2F and ASP-1R as primers and 1 μ g of total RNA. The parameters for PCR were 25 cycles of heating at 94 °C for 1 min, at 55 °C for 2 min and at 72 °C for 3 min. The PCR product was analyzed by 2% agarose gel electrophoresis and blotted to a GeneScreen Plus membrane (Du Pont, Boston, MA, U.S.A.). The blots were hybridized with CS-ACS2, CS-ACS3 or CS-ACS4 probe. Hybridization conditions were the same as described above.

The following pairs of oligonucleotides were used to amplify the CS-ACS2 fragment: CS-ACS2-specific primers CS2-S334 (5'-GGAGGAAAACCTGTGAGGGAGAAGGG-3') and CS2-A776 (5'-GATTGTGGACCGTTGGATCGTTGCT-3'), spanning positions 334 to 358 and 776 to 790 of the CS-ACS2 sequence. Total RNA was treated with RNase-free DNase I (FPLCpure Deoxyribonuclease I; Amersham Pharmacia Biotech, Tokyo, Japan) to eliminate residual genomic DNA. RT-PCR was performed with a GeneAmp RNA PCR Kit (Perkin-Elmer Japan Co. Ltd., Urayasu, Japan) using 1 μ g of total RNA. The parameters for PCR were 18 cycles of heating at 95 °C for 30 sec, at 65 °C for 30 sec and at 72 °C for 1 min. The number of cycles in the PCR reactions was adjusted so that the amplification of products remained in the linear phase.

The PCR products were analyzed by 2% agarose gel electrophoresis and blotted to GeneScreen Plus membranes (Du Pont, Boston, MA, USA). The blots were hybridized with a CS-ACS2 probe. Hybridization conditions were the same as described above.

RNA Gel Blot Analysis

Poly (A)+RNA was isolated from the apices of 10-day-old (the first leaf stage), 15-day-old (the two-leaf stage), 20-day-old (the three-leaf stage) and 25-day-old (the four-leaf stage) of cucumber plants (cv. Rensei, Ougonmegami 2-gou and Shimoshirazu). Poly (A)+RNA (1 μ g per lane) was subjected to electrophoresis on a 1.17% agarose gel that contained 0.66 M formaldehyde and transferred to a GeneScreen Plus membrane (Du Pont, Boston, MA, U.S.A.) by capillary action with 10x SSC as recommended by the manufacturer. After baking at 80°C, the membrane was preincubated in 1 M NaCl, 1% SDS and 10% (w/v) dextran sulfate (sodium salt) at 60°C for 1 h. The denatured ³²P-labeled probe and denatured salmon-sperm DNA were then added to the prehybridization solution, and the membrane was incubated at 60°C for 18 h. Post-hybridization washes were performed twice successively for 5 min each with 2x SSC at room temperature and once for 30 min with 2x SSC, 1% SDS at 60°C. The washed membrane was subjected to autoradiography with an intensifying screen. Then the membranes were washed with boiling 0.01x SSC, 0.01% SDS to dehybridize the probe and blots were rehybridized using an actin gene to ensure that equal amounts of mRNA were present in each lane.

Sex Expression in Flowers

To study the timing of conversion of the first female flower from the female to the male sex after treatment with AVG, the apices of the seedlings (cv. Rensei) were treated with 100 μ M AVG in 0.2% (v/v) Tween 20 for 3 days at 9, 14, and 19 days after planting, respectively. After growth of the seedlings, the sex of each flower on the first 14 nodes was then examined and classified as male or female. A node was designated male if it had at least one male flower and it was designated female if only female flowers were present on it.

Quantification of Ethylene

The apices of 15-day-old seedlings of gynoecious cucumber (cv. Rensei) were treated with or without a 100 μ M AVG in a 0.2% (v/v) Tween 20 solution. The apex was treated by putting a piece of absorbent cotton that had been soaked in the appropriate solution on the apex of the cucumber plant. After application of AVG or the vehicle for 3 h, five apices were excised from plants and incubated for 2 h in a 22.5-ml vessel that was sealed with a silicon stopper. After incubation, a 2-ml sample of gas was removed from the vessel with a syringe that was inserted through the silicon stopper and the ethylene content of the sample was measured with a gas chromatograph (GL-380; GL Sciences Inc., Tokyo, Japan), which was equipped with a flame ionization detector and an alumina column. The instrument was calibrated with a standard sample of ethylene (212 μ l / l).

Results

Isolation and Characterization of Putative cDNAs for ACC Synthases

A set of mixed oligonucleotides was prepared by reference to the conserved amino acid sequence found in ACC synthases (Dong *et al.* 1991, Nakagawa *et al.* 1991, Kende 1993). A cDNA fragment for ACC synthase was amplified by RT-PCR with the mixed oligonucleotides and total RNA isolated from apices of 20-day-old seedlings of monoecious cucumber plants (*C. sativus* L. cv. Ougonmegami 2-gou). The resultant amplified DNA (491 bp) was isolated, subcloned and sequenced. A homology search revealed that this sequence exhibited strong homology to sequences of previously reported ACC synthases both at the nucleotide and the amino acid level. Therefore, the gene was designated *CS-ACS2*. The full length clone encoding *CS-ACS2* was isolated by RACE method. Figure I-1 shows a complete nucleotide sequence of the cloned cDNA and the amino acid sequence deduced from the nucleotide sequence. The *CS-ACS2* sequence is 1761 bp and contains an open reading frame of 445 amino acid with a calculated molecular mass of 50 kDa. The protein shares the invariant 11 residues conserved between ACC synthases and various aminotransferases (Fig. I-1). The dodecapeptide sequence of the active site is also present (Fig. I-1). As shown in Figure I-2, the amino acid sequence deduced from *CS-ACS2* exhibited a high degree of homology (78% identity) to that encoded by the gene (*ST-ACS2*) for ACC synthase from potato (Destefano-Beltran *et al.*, 1995).

I also have cloned two other putative cDNA fragments that encode ACC synthases (Fig. I-3). *CS-ACS3* was amplified by RT-PCR with the

mixed oligonucleotides and total RNA isolated from auxin treated immature cucumber fruit and the deduced amino acid sequence showed a significant homology (95% identity) to the auxin-induced winter squash CM-ACS2 polypeptide (Nakagawa *et al.* 1991). CS-ACS4 was amplified by RT-PCR with the mixed oligonucleotides and total RNA isolated from wounded immature cucumber fruit and the deduced amino acid sequence showed a significant homology (87% identity) to the wound-induced winter squash CM-ACS1 polypeptide (Nakajima *et al.* 1991). The identities of the nucleotide sequence of the CS-ACS2 to the CS-ACS3 and CS-ACS4 were 67% and 64%, respectively.

Figure I-4 presents a result of DNA blot analysis of a RT- PCR reaction on total RNA isolated from apices of 25-day-old seedlings (the four-leaf stage) of gynoecious cucumber (cv. Rensei). The sex of the first female flower was already determined in Rensei at this stage. Since only *CS-ACS2* mRNA transcript was detected at the apices of gynoecious cucumber (Fig. I-4), more detail expression analysis was examined about *CS-ACS2*.

Time Course of the Expression of *CS-ACS2* Gene at the Apex

Figure I-5 shows the patterns of sex expression in flowers of the three cucumber cultivars, Rensei, Ougonmegami 2-gou and Shimoshirazu. Rensei is a gynoecious cucumber cultivar and it formed female flowers on every node examined. Although Ougonmegami 2-gou and Shimoshirazu are monoecious, plants of the two cultivars formed female flowers on different parts of the stems. The first female flowers were formed earlier on Ougonmegami 2-gou than on Shimoshirazu.

The number of genes that corresponded to *CS-ACS2* in three

cucumber cultivars (cv. Rensei, Ougonmegami 2-gou and Shimoshirazu) was estimated by genomic DNA gel blot analysis (Fig. I-6). When the CS-ACS2 probe was allowed to hybridize to *EcoRV* or *SacI* restriction fragments, two bands were observed in the case of *EcoRV* fragments and a single band was observed in the case of *SacI* fragments from all three cultivars.

Figure I-7 shows the time course of the expression of *CS-ACS2* transcripts in the apices of the three cultivars. Although the *CS-ACS2* transcript was not detectable at the apices of 10-day-old Rensei seedlings, it was detected at the apices of 15-day-old plants and its level increased up to 25 days after planting. In the case of Ougonmegami 2-gou, the transcript was detected at the apices of 20-day-old plants and the level was lower in 25-day-old plants. The transcript was barely detectable only at the apices of 25-day-old Shimoshirazu plants. These results indicated that the levels of the synthesis of *CS-ACS2* transcript at the apex were correlated with the formation of female flowers on the stems of the three cucumber cultivars (Figs. I-5 and I-7).

Timing of Sex Conversion of the First Flower from Female to Male by AVG

The apices of gynoecious plants (Rensei) were treated with a solution of AVG, an inhibitor of ACC synthase, at various stages of growth. As shown in Figure I-8, no male flowers were induced on plants that were treated with AVG at 9 days after planting, as was the case with control plants. When the apices of 14-day-old plants were treated with AVG, male flowers were induced on lower nodes including the first flower. The application of AVG to the apices of 19-day-old plants failed to change the

sex of flowers on lower nodes but the plants produced male flowers on higher nodes as compared to the plants that were treated with AVG at 14 days after planting. These results indicate that the sex of flower buds of lower nodes at the apices of 19-day-old plants was already determined and that of flower buds of higher nodes differentiating at the apices was not determined yet and could be changed by the treatment of AVG.

To examine the effect of AVG on the production of ethylene, the amount of ethylene evolved from the apices was measured using apices cut from Rensei seedlings that had been treated with or without AVG for 3 h. As shown in Table I-1, the rate of ethylene production by control apices was $1.36 \text{ nl g}^{-1} \text{ h}^{-1}$, and that by apices treated with AVG was $0.17 \text{ nl g}^{-1} \text{ h}^{-1}$. These results showed that inhibition of ethylene production from AVG-treated apices was correlated with the changes of female flowers to male flowers on the first six to eight nodes (Fig. I-8).

Localization of the Expression of *CS-ACS2* in the Apices of Cucumber Plants

As the apices included young floral buds and unexpanded leaves, the localization of the expression of *CS-ACS2* in the apex was examined using a gynoecious cultivar, Rensei, and a monoecious cultivar, Shimoshirazu. As shown in Table I-2, the Rensei plants produced only female flowers. Although the Shimoshirazu is a monoecious cultivar (Fig. I-5), the plants produced only male flowers on the lower nodes (Table I-2). I examined the expression of *CS-ACS2* at floral buds and at unexpanded leaves that differentiated in the apices of 25-day-old Rensei and Shimoshirazu plants. Although the expression of *CS-ACS2* was barely detected at the unexpanded leaves of both Rensei and Shimoshirazu and at

the floral buds of Shimoshirazu, the *CS-A CS2* mRNA was only detected at the floral buds of Rensei (Fig. I-8).

Discussion

In cucumber plants, it has been suggested that sex expression in flowers is regulated by levels of ethylene at the apex (Rudich *et al.* 1972, 1976; Trebitsh *et al.* 1987). These results suggested that the regulation of ethylene production at the apex is important for the determination of sex expression in flowers of cucumber plants.

I have isolated a cDNA for an ACC synthase from the apices of seedlings of monoecious cucumber (cv. Ougonmegami 2-gou). The sequence of isolated cDNA, designated CS-ACS2, was different from those of cDNA fragments for CS-ACS3 and CS-ACS4 isolated from auxin-treated and cut-injured cucumber tissues, respectively (Figs. I-1 and I-3). Although, the expression of CS-ACS3 and CS-ACS4 was not detected, CS-ACS2 mRNA transcript was detected at the apices of 25-day-old gynoeocious cucumber (Fig. I-4).

I examined the time course of the expression of the CS-ACS2 transcript at the apices of three cucumber cultivars, namely, Shimoshirazu, Ougonmegami 2-gou and Rensei, with different patterns of sex expression in flowers (Figs. I-5 and I-7). The results of genomic DNA gel blot analysis (Fig. I-6) suggested that the gene that corresponded to CS-ACS2 was present as a single or two copies in the genome of all cultivars examined, although interpretation of the data was complicated by the presence of introns. The levels of CS-ACS2 mRNA at the apices of all cultivars seemed to be developmentally regulated. The CS-ACS2 transcript was not detected at the apices at the early stages of development but its synthesis was induced at a later stage. The timing of temporal regulation depended on the cultivar. These results showed that both the timing and the levels of expression of the CS-ACS2 transcript at the apices of the three

cucumber cultivars were correlated with the development of female flowers on the nodes (Figs. I-5 and I-7). Rudich *et al.* (1976) reported that the rate of evolution of ethylene from apices of gynoecious cucumber plants increased dramatically from 8 to 24 days after germination while the increase in the rate of evolution of ethylene was observed in monoecious cucumber only after 21 days of growth. These patterns of evolution of ethylene resembled the patterns of expression of the *CS-ACS2* gene at the apices of cucumber plants. The treatment of apices of gynoecious cucumber plants (cv. Rensei) with AVG, an inhibitor of ACC synthase (Adams and Yang 1979, Boller *et al.* 1979), caused a decrease in ethylene production at the apices and plants treated with AVG produced male flowers instead of female flowers (Table I-1 and Fig. I-8). As shown in Figure I-6, the conversion of female flowers to male flowers at the lower nodes was caused by the application of AVG for 3 days to apices of 14-day-old gynoecious plants (Rensei). This result suggests that endogenous ethylene acts on floral buds that are differentiating at the apex to form female flowers at this stage of growth. In fact, as shown in Figure I-7, the *CS-ACS2* transcript was induced at the apices of the seedlings (Rensei) 15 days after planting. The timing of the action of ethylene on the formation of female flowers coincided with the timing of induction of the *CS-ACS2* transcript at the apex. These results suggest the ACC synthase encoded by *CS-ACS2* is potentially involved in regulation of the development of female flowers at the transcript level at the apex.

The expression of *CS-ACS2* was localized in the apices of cucumber plants. Although expression of the *CS-ACS2* was barely detected at the unexpanded leaves of both Rensei and Shimoshirazu plants and at the floral buds of Shimoshirazu plants that would develop into male flowers, the *CS-ACS2* mRNA was remarkably detected at the floral buds of Rensei

plants that would develop into female flowers (Table I-2 and Fig. I-9). These results suggest that the *CS-ACS2* transcript is expressed only in limited floral buds that will develop into female flowers. In fact, ethylene was thought to act on a floral bud to induce the development of a female flower. Potentially male buds detached from plants at the bisexual stage reverted to female buds upon addition of auxin *in vitro* (Galun *et al.* 1962, 1963) and it was clarified that the feminizing effect of auxin is mediated by ethylene (Shannon and De La Guardia 1969 and Trebitsh *et al.* 1987). These results suggested that the expression of *CS-ACS2* transcript at floral buds induces the development of female flowers *via* ethylene formation.

Table I-1. The effects of the application of AVG to apices on the production of ethylene by the apices of cucumber plants (cv. Rensei)

Treatment	Ethylene production nl/g·h \pm SE
control	1.36 \pm 0.25
AVG	0.17 \pm 0.03

The apices of 15-day-old cucumber plants (cv. Rensei) were treated with (AVG) or without (control) 100 μ M AVG for 3 h. After the treatment, 5 apices were excised from plants and placed in a gas-tight vessel for 2 h. Results for 5 groups of 5 plants (\pm SE) are given.

Table I-2 Sex expression in the gynoecious (cv. Rensei) and monoecious (cv. shimoshirazu) cucumber plants.

Cultivar	Number of nodes per plant		
	Female	Male	Aborted
Rensei	17.4 ± 0.2	0.0 ± 0.0	2.6 ± 0.2
Shimoshirazu	0.0 ± 0.0	18.8 ± 0.2	1.2 ± 0.2

The sex of each flower on the first 20 nodes of the main stem was examined and classified as male or female. Results for 5 plants (\pm SE) are given.

Figure I-1. Nucleotide sequence of CS-ACS2 cDNA and its deduced amino acid sequence. The deduced amino acid residues are given in the single-letter code. The amino acid sequence corresponding to the active site of ACC synthase is underlined. The eleven boxed amino acids are the invariant residues conserved between ACC synthases and various aminotransferases.

Figure I-2. Comparison of the deduced amino acid sequence of CS-ACS2 and the amino acid sequence of potato ST-ACS2. Amino acids are written by the single-letter code. The identity is indicated by a box. Gaps are incorporated to obtain a maximum matching of sequences and are shown in dashes.

CS-ACS2 1: MAIEIEIEQNSSVELSRIGTSETHGEDSPYFAGWKAYDEDPYNESTNPSGVIQMG LAENQVSFDLLEEYL 70
 ST-ACS2 1: MAIEIE-QRPTVRLSNVAISDTHGEDSPYFAGWKAYDENPFDEVHNPSGVIQMALAENQVSFDLLEEYL 69

CS-ACS2 71: EENCEGEINYLNSGFRENALFQDYHGLFSFRSAMGSFMEIRGGRAKFDFNRVLLTAGATAANELLTFIL 140
 ST-ACS2 70: -EKKKDDIAEISRRENALFQDYHGLVCFRKAMATFMEQVRGGRARFDFDRVYITAGATAANELLTFIL 138

CS-ACS2 141: ANPGDALLVPTPYYPGFDRDLRWRTGVKIVPIHCDSNNFQIIPKALEEAYNSATEMKIKVRGVLITNPS 210
 ST-ACS2 139: ADPGDALLVPTPYYPGFDRDLRWRTGVKIIIVHCDSNNFQVILQALEEAYKDAESNNIKVRGVLITNPS 208

CS-ACS2 211: NPLGATIQRSTIEDILDFVTRKNIHLVSDEIYSGSVFSSDEFTSVAEVLSESRGYNAERVHIVYSLSKDL 280
 ST-ACS2 209: NPLGTTIVQRCVLEILEFVARKNIHLVSDEIYSGSAFCCSEFVSAEILESRNYKDSERVHIVYSLSKDL 278

CS-ACS2 281: GLPGFRVGTIYSYNDKVVTTARRMSSFTLISSQTRFLASMLSNRKFTENYIKMNRDLKKRYEMITEGL 350
 ST-ACS2 279: GLPGFRVGTIYSYNDKVVTTARRMSSFTLISSQTOQLASMLSDKFTENYIKNRRLRRRYEMITEGL 348

CS-ACS2 351: RTAGIECLEGNAGLFCWMNLSPLLKDKKTKEGEIEIKRILKEVKLNISPGSSCHCSEPGWFRVCFANMS 420
 ST-ACS2 349: RSAGIECLRGNAGLFCWMNLSLLEKPTKECELEVNNTILHEVKLNISPGSSCHCSEPGWFRVCFANMS 417

CS-ACS2 421: EKTLLHVALDRIRRFMERMKKENEAN 445
 ST-ACS2 418: ENTLEIALKRHHFMETRGILQKY- 441

Figure I-3. Nucleotide sequences of CS-ACS3 (A) and CS-ACS4 (B) cDNAs and their deduced amino acid sequences. The deduced amino acid residues are given in the single-letter code. The amino acid sequences that are conserved in ACC synthases are underlined.

(A) CS-ACS3

1 CGCATTCAAAAAGGCATTGGTAGAATTTATGGCGGAAATTAGAGGAAACAAAGTAACATTTGAAGCAAAT 70
A F K K A L V E F M A E I R G N K V T F E A N

71 AACATAGTCCTTACAGCTGGCGCTACATCAGCCAATGAAACACTTATGTTCTGCCTTGACAGAGGCTGGCG 140
N I V L T A G A T S A N E T L M F C L A E A G D

141 ATGCCTTTCTCCTCCCAACTCCATACTATCCAGGATTTGATAGAGATTTGAAATGGAGAACCGGAGTTGA 210
A F L L P T P Y Y P G F D R D L K W R T G V E

211 GATTGTGCCAATTCATTGCACTAGCTCCAACGGCTTCCAAGTCACCCAACCAGCTTTAGAACAAGCCTAT 280
I V P I H C T S S N G F Q V T Q P A L E Q A Y

281 CAAGAAGCCCAAGCTCGCAACCTACGTGTCAAAGGCGTATTGGTTACC 328
Q E A Q A R N L R V K G V L V T

(B) CS-ACS4

1 AGAGTTTCGAAATGCGATTGCGAGTTTTATGGGGAAGGCGAGGGGTGGGAGGGTGAATTCGACCCAAGT 70
E F R N A I A S F M G K A R G G R V K F D P S

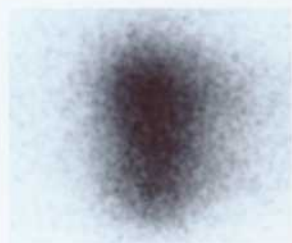
71 CGGATCGTGATGGGCGGCGGTGCTACTGGGGCGAGCGAAGCCGTCATCTTTGTTTAGCCGATCCGGGCG 140
R I V M G G G A T G A S E A V I F C L A D P G D

141 ATGCCTTTTGATTCTTCTCCATATTATGCAGCGTTTATAGAGATCTTCAATGGAGAACACGAGCACA 210
A F L I P S P Y Y A A F D R D L Q W R T R A Q

211 AATAATTCAGTTTATTGCAACAGCTCGAACAACCTTCAAATCACAAGGGAAGCCTTGGAAAGTAGCATAT 280
I I P V H C N S S N N F Q I T R E A L E V A Y

281 AAAAAGGCTGAAGAAAGCAATATCAAAGTAAAGGCTTATAATAACC 328
K K A E E S N I K V K G L I I T

Figure I-4. RT-PCR analysis of expression of *CS-ACS2*, *CS-ACS3* and *CS-ACS4* on total RNA from apices of 25-day-old plants of gynoecious cucumber (cv. Rensei). The PCR reaction was conducted using primers ASP-2F and ASP-1R under the following conditions: 25 cycles of 94°C, 1 min; 55°C, 2 min; 72°C, 3 min. RT-PCR product was separated on an agarose gel, transferred to a nylon membrane, and allowed to hybridize with the *CS-ACS2*, *CS-ACS3* and *CS-ACS4* probes.



CS-ACS2

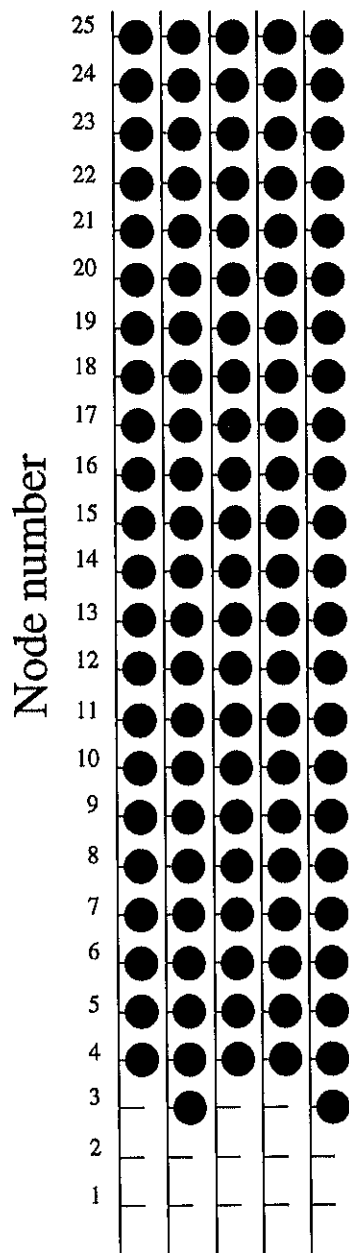


CS-ACS3

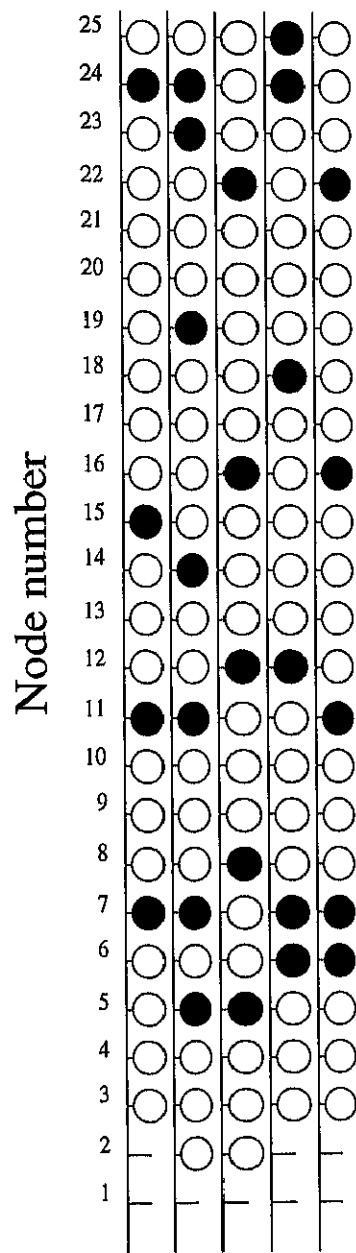


CS-ACS4

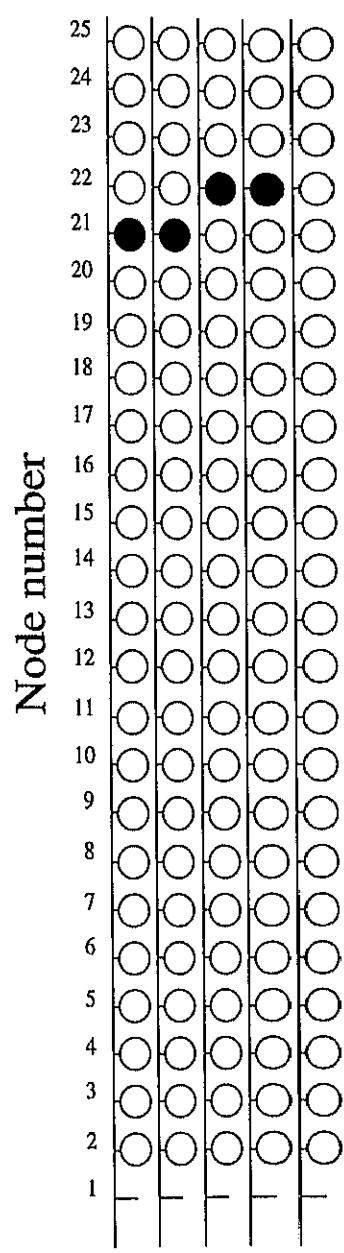
Figure I-5. The patterns of sex expression in flowers of the three cultivars, Rensei, Ougonmegami 2-gou and Shimoshirazu. Plants were grown in soil-filled pots in a growth chamber with 12 h of light per day at 25 °C. The node number indicates the position of individual nodes along the main shoot. Closed circles, nodes with female flowers; open circles, nodes with male flowers; no circles, vegetative nodes. Data from five plants are presented in each case.



Rensei



Ougonmegami 2-gou



Shimoshirazu

Figure I-6. Genomic DNA gel blot analysis with CS-ACS2 cDNA as the probe. The DNA from three cucumber cultivars, Rensei (A), Ougonmegami 2-gou (B) and Shimoshirazu (C), was separately digested with EcoRV and SacI. Fragments were separated on a agarose gel, transferred to a nylon membrane, and allowed to hybridize with the CS-ACS2 probe. The sizes of DNA markers are indicated on the left side.



Figure I-7. Time course of the expression of *CS-ACS2* transcripts at the apices of Rensei, Ougonmegami 2-gou and Shimoshirazu. The plants were grown under the conditions described in Fig. 4. Poly (A)⁺ RNA was extracted from the apices of Rensei (lanes 1-4), Ougonmegami 2-gou (lanes 5-8) and Shimoshirazu (lanes 9-12) at 10-day-old (10), 15-day-old (15), 20-day-old (20) and 25-day-old (25). Extracted poly (A)⁺ RNA (1 μ g) was fractionated on a formaldehyde-containing agarose gel, transferred to a nylon membrane, and allowed to hybridize with the *CS-ACS2* probe.

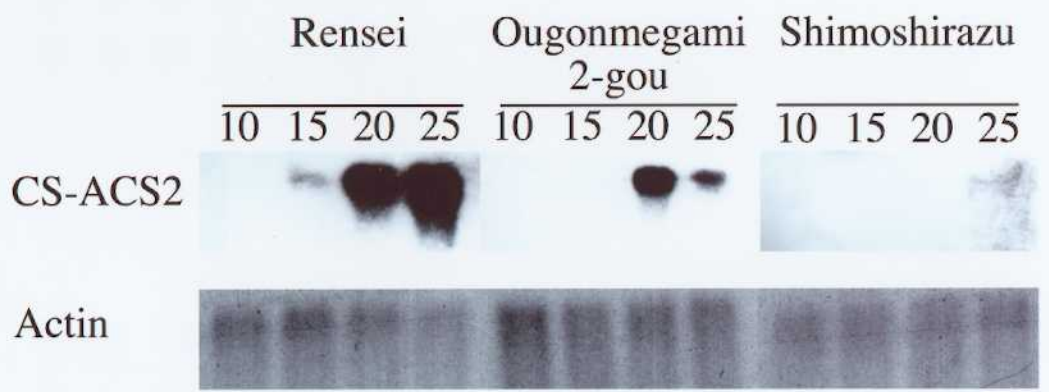
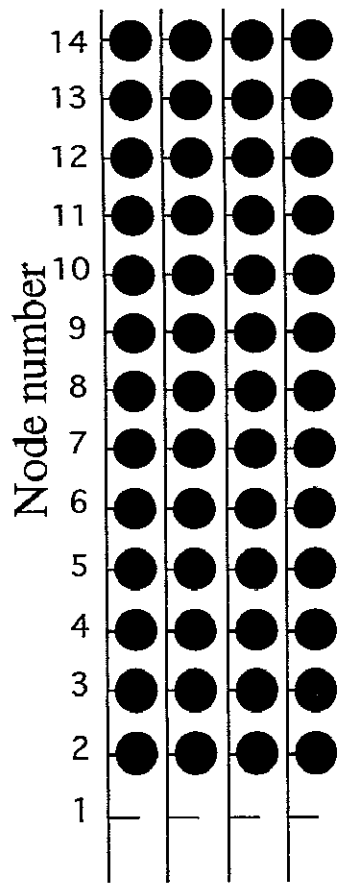
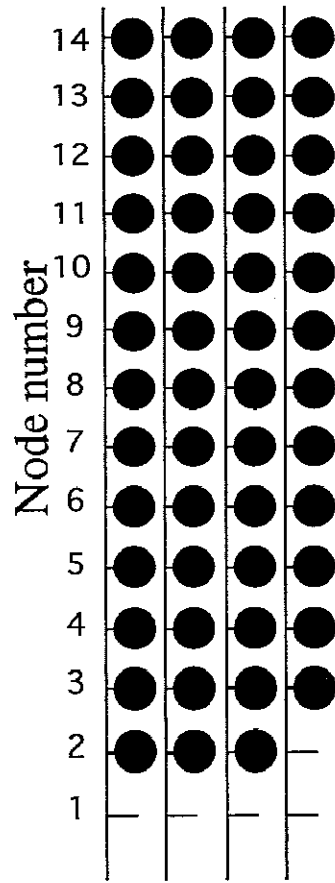


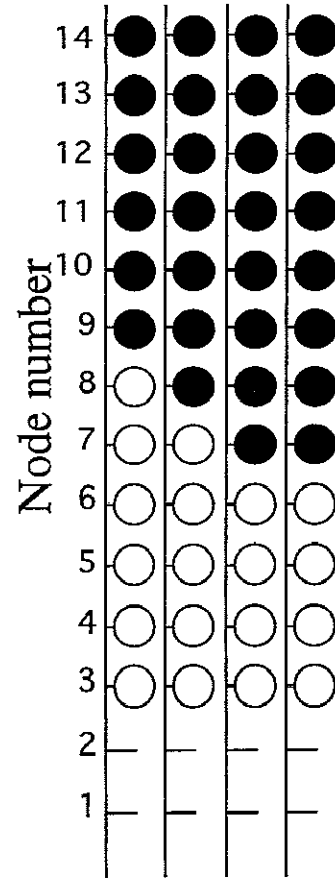
Figure I-8. The timing of sex conversion of flowers from female to male by treatment of apices with AVG. AVG (100 μ M) was applied to the apices of cucumber plants (cv. Rensei) of indicated ages for 3 days. The node number indicates the position of individual nodes along the main shoot. Closed circles, nodes with female flowers; open circles, nodes with male flowers; no circles, vegetative nodes. Data from four plants are presented in each case.



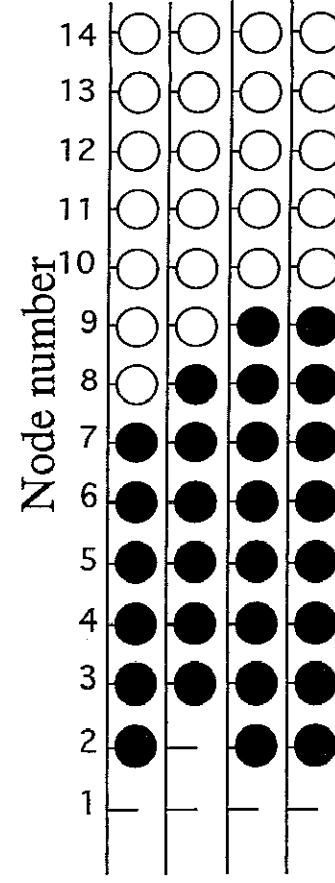
No treatment



9-day-old



14-day-old



19-day-old

Figure I-9. Expression of *CS-ACS2* at floral buds and unexpanded leaves included in the apices of Rensei and Shimoshirazu cucumber plants. Total RNA was extracted from floral buds and unexpanded leaves from the eighth node to the sixteenth node of 25-day-old Rensei and Shimoshirazu plants. *CS-ACS2* cDNA was amplified from the total RNA by RT-PCR using primers CS2-S334 and CS2-A776 under the conditions described in “Materials and Methods”. The PCR products were separated on an agarose gel, transferred to a nylon membrane and allowed to hybridize with the *CS-ACS2* probe.

Shimoshirazu

Leaves

Floral buds



Rensei

Leaves

Floral buds

